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Original Research Article

Study Of Lead Resistant Microorganism and Plant Interaction

Sambit Mukherjee[1], Debpratim Khanna[2] and Sabyasachi Chatterjee[3*]
[1]Department of Biotechnology, The University of Burdwan, Rajbati, Bardhaman - 713 104, West Bengal, INDIA

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Abstract: Environmental pollution with toxic heavy metals is a wide spread problem throughout the world with the advancement of industries and indiscriminate use of toxic metals for various manufacturing processes. Unlike organic compounds, metals cannot be degraded, and cleanup usually requires their removal from their contaminated sites. Conventional physico-chemical methods such as electrochemical treatment, ion exchange, precipitation, reverse osmosis, evaporation and sorption are costly, high-energy consuming and produce toxic waste products which require further disposal. Heavy metal resistant microorganisms and plants have been well established as bio accumulators of soluble and particulate forms of heavy metals. Microbe and plant related technologies may provide an alternate or additional approach to conventional method of heavy metal removal. Therefore, a study was carried out to determine the effect of lead resistant microorganism on heavy metal resistant plant *Helianthus annuus* and newly found *Solanum nigrum* in remediation of lead. Inoculation of the plants with bacterial isolate showed significantly increased growth rate. Heavy metal deposition in the leaves and stem of the plants were tested by scanning electron microscopy.

Keywords: Bioaccumulator, Heavy metal resistant plant and microbe, *Helianthus annuus*, Lead acetate, *Solanum nigrum*, Scanning Electron Microscopy

Introduction

The beginning of the last century ushered in inventions and technical innovations that definitely have paved way for life with sophistication but simultaneously resulted in an unwanted exposure of the biota to numerous physical and chemical lethal substances. An indiscriminate use of such substances has eventually lead to their infiltration and accumulation in various levels of the ecosystem resulting in "hazards to human health, harm to living resources and ecological systems, damage to structure or interference with legitimate uses of the environment" and has been called pollution[1].

Heavy sulfhydryl reactive metals such as lead are potent toxic elements known to bio accumulate and express their toxicity to both the human body and environment. Most toxic substances (organic pollutants, heavy metals) come from anthropogenic activities such as mining, traffic, heavy industry, etc. Contrary to organic pollutants, heavy metals cannot be degraded or destroyed. From a physiological point of view, metals fall into three main categories: (i) those which are essential and basically non-toxic (e.g. Ca and Mg) (ii) essential, but harmful at high concentrations (typically, Fe, Mn, Zn, Cu, Co, Ni and Mo) and (iii) toxic (e.g. Hg or Cd). Further, interactions with metals depend not only on the particular element but also on its chemical speciation i.e. valency[2,3]. Heavy metal toxicity can result in damaged or reduced mental and central nervous function, lower energy levels and damage to blood composition, lungs, kidneys, liver, and other vital organs. Long-term exposure may result in slowly progressing physical, muscular, and neurological degenerative processes that mimic Alzheimer's disease, Parkinson's disease, muscular dystrophy and multiple sclerosis. Allergies are not uncommon, and repeated long-term contact with some metals (or their compounds) may cause cancer.

Not only human beings but both plants and microorganisms[4] are equally affected by heavy metals. Heavy metals, because of their strong ionic nature bind to many cellular ligands and displace native essential metals from their normal binding sites. They also disrupt proteins by binding to sulphydryl groups and nucleic acids by binding to phosphate or hydroxyl groups. As a result DNA and protein conformation change and functions get disrupted. Heavy metals also affect oxidative phosphorylation and membrane permeability of cells.

Thus there is a need to remove heavy metals from contaminated sites. Remediation processes can be expensive, as they are mostly ex-situ methods involving excavation

***Corresponding Author:**
Dr. Sabyasachi Chatterjee,
Department of Biotechnology,
The University of Burdwan,
Rajbati, Bardhaman - 713 104,
West Bengal, INDIA.

of impacted soils and subsequent treatment at the surface. Therefore new efficient, inexpensive and non-environmentally disruptive technologies are still developing. One of the groups of such new technology is called bioremediation, which involves use of heavy metal tolerant plants and microbes to detoxify contaminated sites. Both provide cost-effective[5] and easy ways of detoxifying heavy metal contaminated sites compared to physic-chemical methods such as electrochemical treatments, ion exchange, precipitation, reverse osmosis, evaporation, and sorption[6,7] which are costly[8] high energy consuming and produces toxic waste products which require disposal. In response to heavy metal toxicity microorganisms have developed ingenious mechanisms of lead resistance[9] and detoxification[10,11,12,13,14]. Plants too have certain mechanisms which make them ideal for bioremediation[15,16,17,18]. Plant based bioremediation technologies have been collectively termed as phytoremediation[5], which is an emerging low-cost and ecologically benign technology for decontamination of soils and refers to the use of green plants and their associated micro biota for the in-situ treatment of contaminated soil and ground water through physical, chemical and biological processes. Plants suitable for phytoremediation should have a high biomass production with enhanced metal tolerance and metal uptake potential. Effluents having heavy metals thus can be treated with microorganisms which applies processes like biosorption, bioaccumulation, and bioprecipitation or plants which uses processes such as rhizofiltration, phytotransformation, phytoextraction and phytostabilization[19,20] to recover metals from contaminated soil.

Research shows plants and microorganisms have been applied together for remediation purposes of contaminated soil[15,21,22,23]. The bacteria associated with plant roots may have profound effects on plant growth and nutrition through a number of mechanisms such as N_2 fixation, production of phytohormones and siderophores and transformation of nutrient elements. Improvement of the interactions between plants and beneficial rhizosphere microorganisms can enhance biomass production and tolerance of the plants to heavy metals and are considered is be an important component of phytoremediation technology. Plant species such as Indian mustard (*Brassica juncea*), Sunflower (*Helianthus annuus*) has been widely applied for phytoremediation and show high tolerance to heavy metals and therefore are used in phytoremediation studies[15,16,24,18]. *Solanum nigrum*, a newly found hyperaccumulator has shown the potential to remediate Cd-contaminated soils[25,26,27] as well as metalaxyl, a commonly used persistent, mobile and leachy fungicide[28]. Metal-resistant microorganisms and metal chelators synergistically enhance the phytoremediation efficiency of *Solanum nigrum* in Cd- and Pb-contaminated soil[29].

This present study involves isolation of heavy metal tolerant microorganisms from soil of Durgapur Steel Plant and to study plant-microbe beneficial association.

Materials and Methods

Sample collection:

Soil sample was collected from the 'Solid Waste Dumping Zone of DSP (Durgapur Steel Plant) under SAIL (Steel Authority Of India Limited) located at Durgapur town of Burdwan district of West Bengal, India. Sample was specifically collected from those areas with visible flora growth for chances of getting more heavy metal resistant bacteria.

- The sample was collected in March, 2013 in a sterilized plugged conical flask and was taken to laboratory to be maintained at 4°C for further studies.

Isolation of bacteria from the soil sample:

- Bacteria were isolated from the soil sample by growing them on sterilized Luria-Bertani (LB) Agar plates.
- The soil sample was suspended in distilled water (1g in 10ml) and was diluted upto 10^{-5} dilution. 1ml of each dilution was used to pour plate on the LB-Agar plates and was incubated at 37°C for 48 hours.
- The most dominant colonies in terms of size were then chosen for the next phase of isolation.

Isolation of lead (Pb) resistant bacteria:

- To obtain lead (Pb) resistant strains out of all the isolated bacteria, the dominant colonies in terms of size were chosen and then every colony was inoculated in LB broth tubes supplemented with 1mM lead acetate. The tubes were incubated at 37°C for 48 hours.

- After incubation growth was observed in five tubes. One loopful of culture from each tube was then streaked over LB-Agar slants and incubated at 37°C for 48 hours. Out of those, the bacterial colony showing highest growth was chosen for our study. The isolate was named as SSD1, according to the name of the persons who isolated it. The slants were then stored at 4°C for further use.

Determination of minimal inhibitory concentration of lead on SSD1:

- MIC (Minimal inhibitory concentration) was determined by growing the bacterial isolate on LB Agar plates supplemented with 1mM-10mM lead acetate. MIC was noted on the point of highest concentration beyond which SSD1 failed to grow even after 10 days of optimal condition of incubation.

Growth profile of SSD1:

Two conical flasks containing 50ml LB broth were taken. One was supplemented with 5mM lead acetate solution and the other was kept as control. One loopful of SSD1 was inoculated in both the conical flasks and incubated at 120 rpm in a shaker incubator at 37°C. Optical density was measured at 620 nm after every 1 hour intervals.

Determination of SSD1 biomass for different concentrations of lead acetate:

One loopful of bacterial isolate (SSD1) was inoculated in LB broth supplemented with different concentrations of lead acetate solutions (3mM, 3.5mM, 4mM, 4.5mM and 5mM) and incubated at 37°C for 24hours. 2ml of each culture was taken and dry biomass was calculated.

Biochemical characterization of the lead resistant bacterial isolates:

Biochemical characterizations of the isolates were done as shown in Table 2-6.

Experimental setup for a comparative study of bioremediation properties of SSD1 and the plants:

In order to determine presence of bioremediation properties an experimental setup with SSD1 and two plants, *Helianthus annuus* and *Solanum nigrum* was designed. 10ml of 5mM lead acetate solution was added to the sterilized soil in which plants were grown and was watered with MiliQ throughout the experiment. 20ml each of SSD1 was added to both the plants as per Table 1. The experimental setup was maintained for 30 days to study phenotypic changes. Later the plants and plant parts were sacrificed for Scanning Electron Microscopic analysis.

Classification:

Solanum nigrum

Helianthus annus

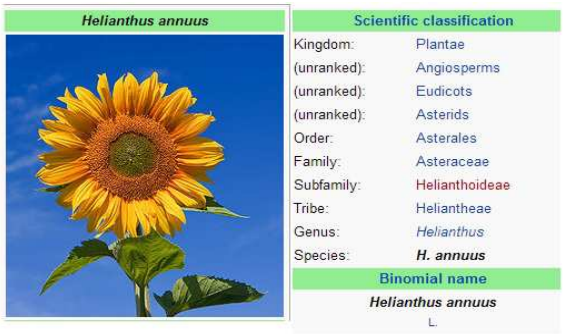


Table.1: Application of lead acetate and SSD1 to each experimental setup

PLANT	LEAD ACETATE (5mM, 10ml)	SSD1 (20ml)
Helianthus annuus A (CONTROL)	×	×
Helianthus annuus B	✓	×
Helianthus annuus C	✓	✓
Solanum nigrum A (CONTROL)	×	×
Solanum nigrum B	✓	×
Solanum nigrum C	✓	✓

Effect of minimal media on SSD1:

SSD1 was streaked over M9 media and incubated at 37°C for 48 hours.

Effect of temperature on SSD1:

Two set of LB broth containing tubes were taken. One set was supplemented with 5mM lead acetate solution and the other was kept as control. One loopful of SSD1 was inoculated in all the tubes. The tubes were then incubated at different temperatures like 10°C, 20°C, 30°C, 37°C, 45°C and 60°C for 24 hours. Optical density (O.D.) was taken at 620 nm after incubation.

Effect of pH on SSD1:

Two set of LB broth containing tubes were taken. One set was supplemented with 5mM lead acetate solution and the other was kept as control. One loopful of SSD1 was inoculated in all the tubes. pH was adjusted like 2, 4, 6, 7, 8, 10 with 1N HCl and 1N NaOH. All the tubes were incubated at 37°C for 24 hours and O.D. was taken at 620 nm after incubation.

Polysaccharide test:

Overnight grown cultures with lead acetate and without lead acetate were harvested by centrifugation at 10,000 rpm for 10 minutes. Supernatant was separated in another tube and kept at 4°C for further use. The pellet was collected, washed with MiliQ water and kept in water bath at 70°C for 15 minutes. Then 2-3 drops of 1N HCl was added, heated for 5 minutes and centrifuged at 10,000 rpm for 5 minutes. The supernatant was taken and double volume of 70% ethanol was added to both the tubes. Both the tubes were kept at 4°C for overnight. Since white precipitate was not observed after 24 hours confirming the absence of any polysaccharide, so Dubois method was not performed.

Results

Bacterial growth:

Fig.1: 10^{-1} dilution

Fig.2: 10^{-2} dilution

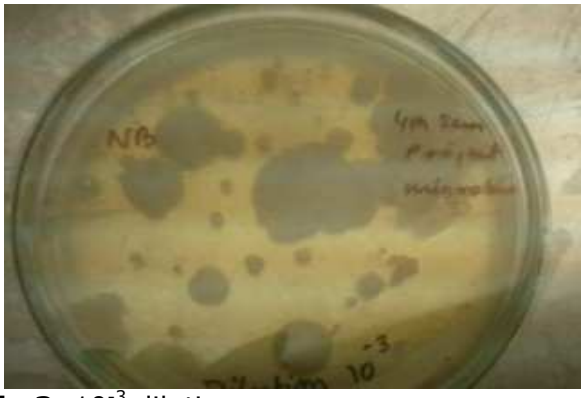


Fig.3: 10^{-3} dilution

Fig.4: 10^{-4} dilution

Fig.5: 10^{-5} dilution

Genomic DNA isolation:

Two LB broth containing tubes were inoculated with one loopful each of SSD1. One tube was supplemented 5mM lead acetate solution and the other was kept as control. Both were incubated at 37°C for 24 hours. Genomic DNA isolation was performed by following Vincent and Janarthanan's protocol[30].

Table.2: Bacterial biomass (SSD1) production with different concentrations of lead acetate:

Concentration of lead (mM)	Weight of empty tube(gm.)	Weight of tube with biomass(gm/ 2ml)	Weight of net biomass(gm.)
3	**1.073**	**1.100**	**0.027**
3.5	1.073	1.095	0.022
4	1.073	1.093	0.020
4.5	1.073	1.090	0.017
5	**1.073**	**1.066**	**0.007**

Table.3: Biochemical characterization:

Biochemical Test	Isolate 1	Isolate 2	Isolate 3	Isolate 4 (SSD 1)	Isolate 5
Amylase Test	-ve	-ve	-ve	**-ve**	-ve
Catalase Test	++ve	++ve	+ve	**-ve**	+ve
VP Test	-ve	-ve	-ve	**-ve**	-ve
Indole Test	-ve	-ve	-ve	**+ve**	-ve
Oxidase Test	-ve	-ve	-ve	**-ve**	-ve

"+ve & -ve" indicates efficient growth and no response respectively.

Table.4: Acid Gas Production Test:

Samples	Maltose		Sucrose	
	Acid	Gas	Acid	Gas
Isolate 1	+ve	-ve	-ve	-ve
Isolate 2	+ve	-ve	-ve	-ve
Isolate 3	+ve	-ve	-ve	-ve
Isolate 4 (SSD1)	**+ve**	**-ve**	**+ve**	**-ve**
Isolate 5	+ve	-ve	-ve	-ve

"+ve & -ve" indicates efficient growth and no response respectively.

Biochemical Tests:

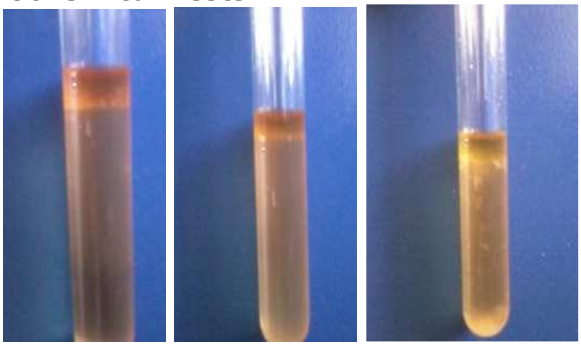


Fig.6: Indole Test (Isolate 1)
Fig.7: Indole Test (Isolate 2)
Fig.8: Indole Test (Isolate 3)

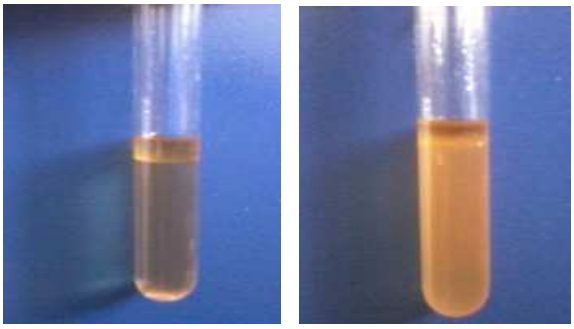


Fig.9: Indole Test (Isolate 4, **SSD1**)
Fig.10: Indole Test (Isolate 5)

Fig.11: Oxidase Test (Isolate 1-5)

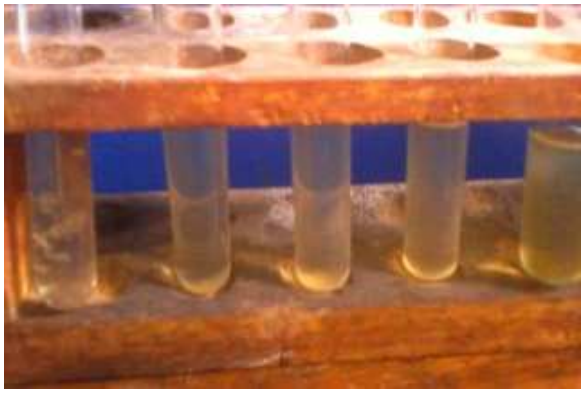


Fig.12: VP Test (Isolate 1-5)

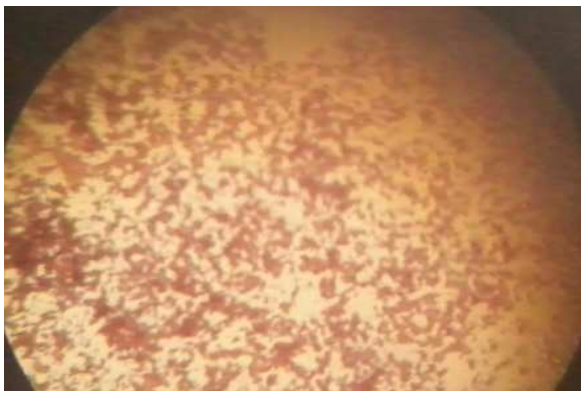


Fig.13: Gram Staining (Isolate 1, 40x)

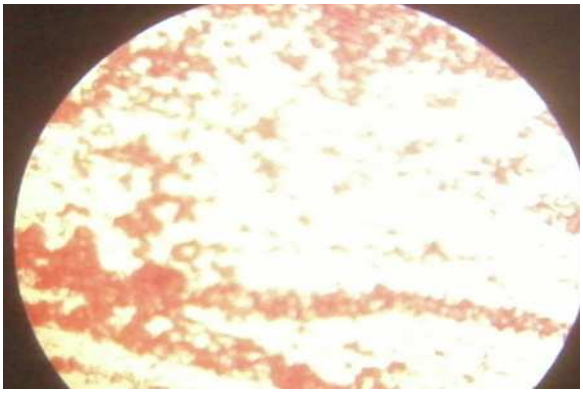


Fig.14: Gram Staining (Isolate 2, 40x)

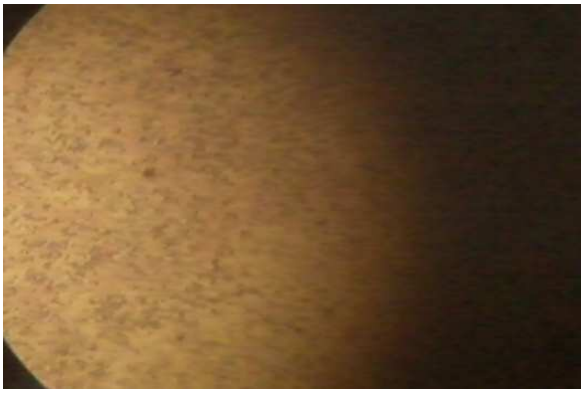


Fig.15: Gram Staining (Isolate 3, 40x)

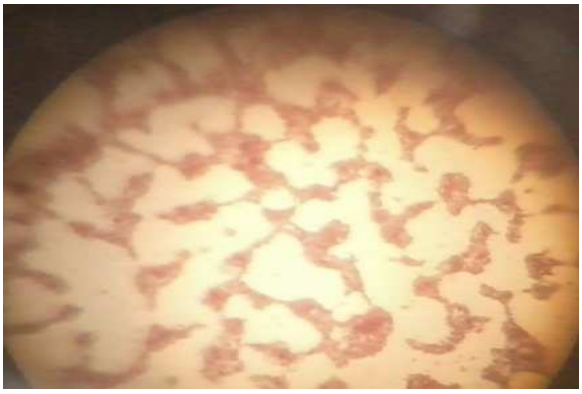


Fig.16: Gram Staining (Isolate 4, **SSD1**, 40x)

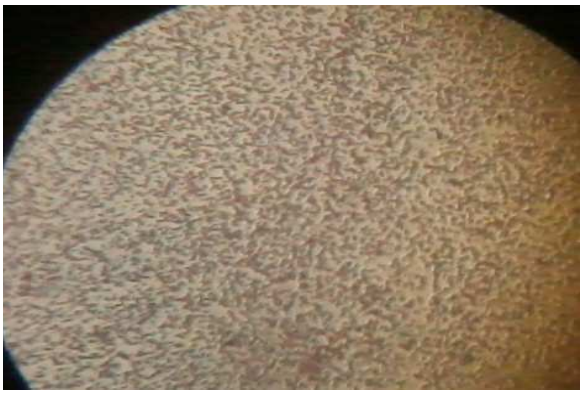


Fig.17: Endospore Staining (Isolate 1, 40x)

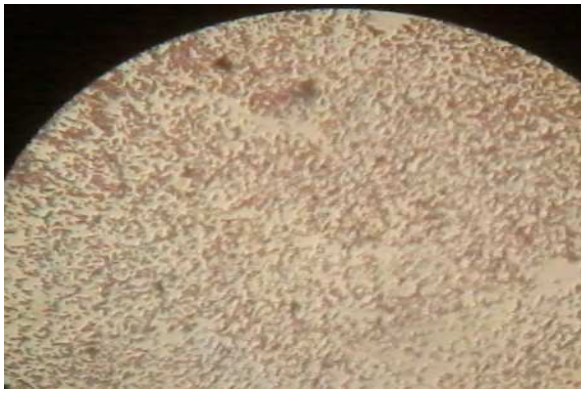


Fig.18: Endospore Staining (Isolate 2, 40x)

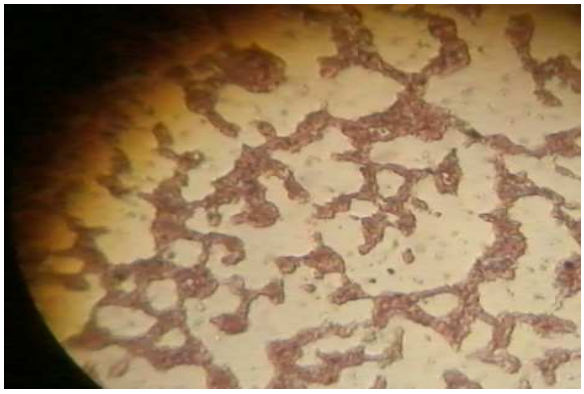


Fig.19: Endospore Staining (Isolate 3, 40x)

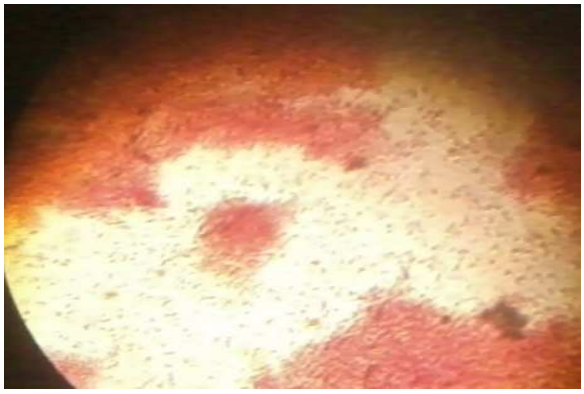


Fig.20: Endospore Staining (Isolate 4, **SSD1**, 40x)

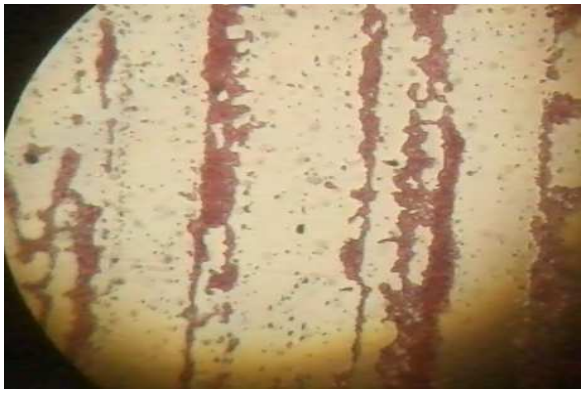


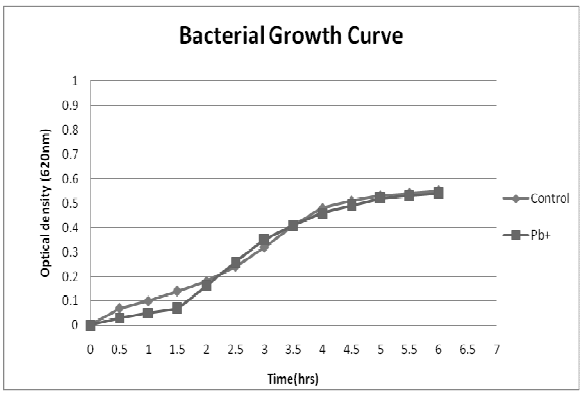
Fig.21: Endospore Staining (Isolate 5, 40x)

Table.5: Gram Staining

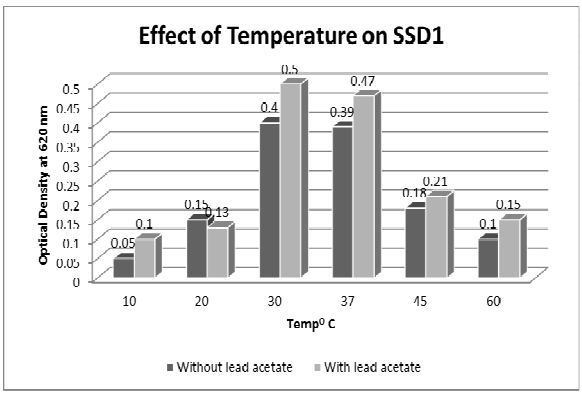
Sample	Result	Shape
Isolate 1-pinkish yellow colony	Gram -ve	Strepto Cocci
Isolate 2-yellow colony	Gram -ve	Strepto Cocci
Isolate 3-greenish white colony	Gram -ve	Cocci
Isolate 4 (SSD1)-white colony	Gram -ve	Strepto Cocci
Isolate 5-pinkish white colony	Gram -ve	Strepto Cocci

Table.6: Endospore Staining

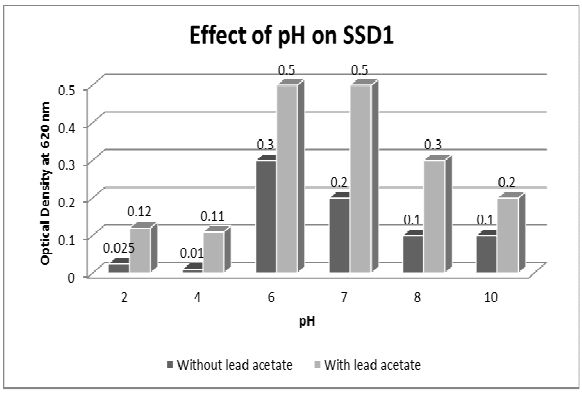
Sample	Result
Isolate 1	-ve
Isolate 2	-ve
Isolate 3	-ve
Isolate 4 (SSD1)	+ve
Isolate 5	-ve



Graph.1: SSD1 growth curve with 5mM lead acetate (Pb+) and control



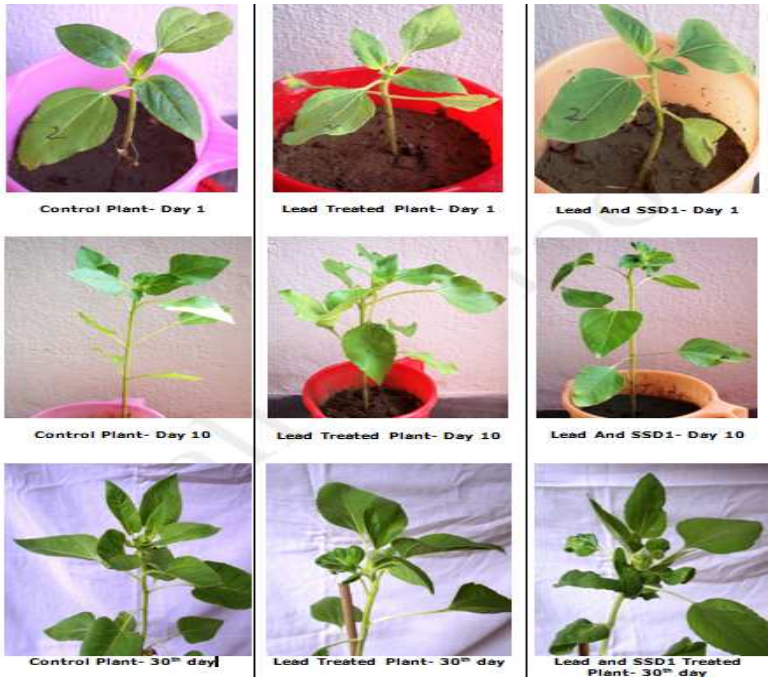
Graph.2: Effect of Different Temperature on SSD1



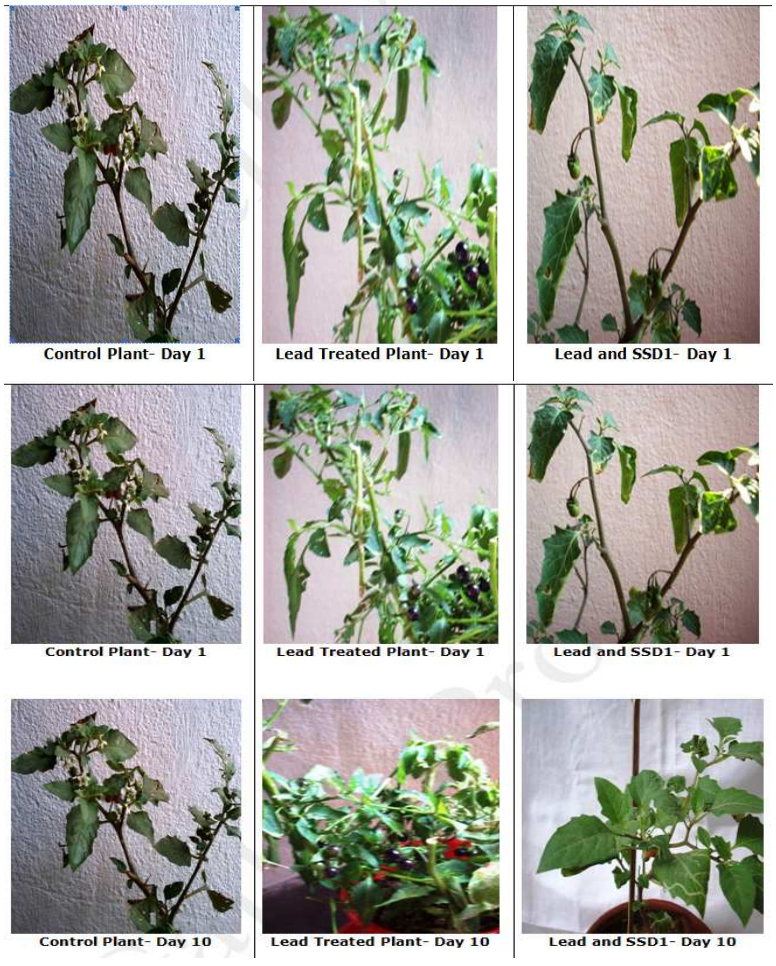
Graph.3: Effect of Different pH on SSD1

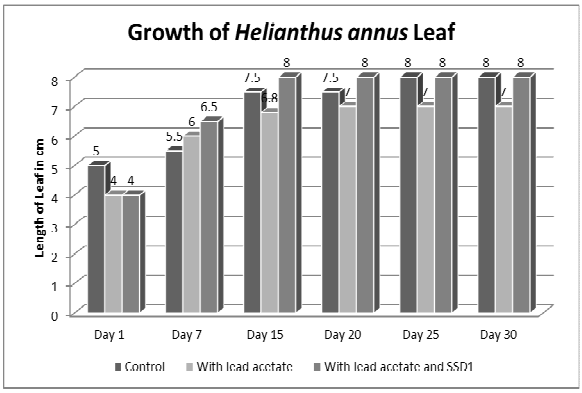
Plate.1: Figures of Phenotypic Changes in the Plants

***Helianthus annuus*:**

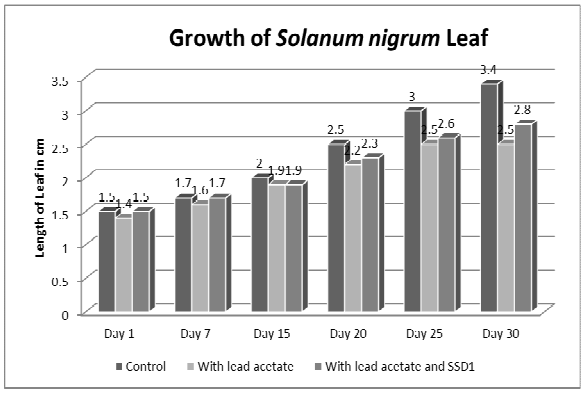


***Solanum nigrum*:**

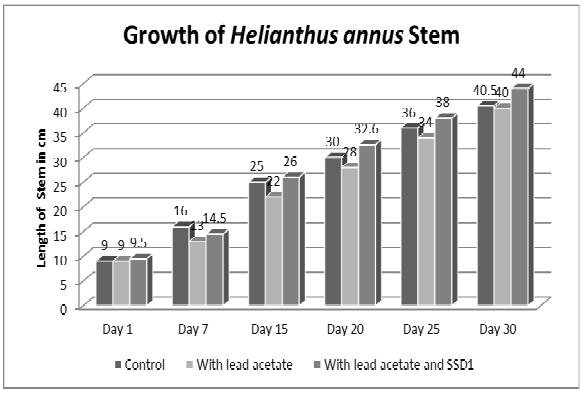




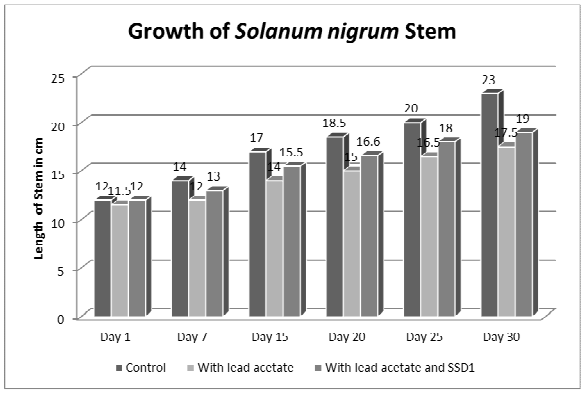
Graph.4: Growth of *Helianthus ann*us Leaf



Graph.6: Growth of *Solanum nigrum* Leaf



Graph.5: Growth of *Helianthus annus* Stem



Graph.7: Growth of *Solanum nigrum* Stem

Table.7: Morphological Characteristics of *Helianthus annus* and *Solanum nigrum*

Helianthus annus:

Time	Treatment	Phenotypic changes
Day 1	Control 5mM Lead acetate 5mM lead acetate and SSD1	Healthy plants with spotless leaves and stem
Day 7	Control 5mM lead acetate 5mM lead acetate and SSD1	No visible changes
Day 15	Control	Normal plant with no changes
	5mM lead acetate	Slightly yellow leaves, growth of shoot apex not normal
	5mM lead acetate and SSD1	Slight bending of shoot apex, loosing leaf symmetry.
Day 20	Control	Normal plant
	5mM lead acetate	Yellow leaves, abnormal growth at shoot apex.
	5mM lead acetate and SSD1	Curling of leaves, loss of leaf symmetry
Day 25	Control	Normal plant
	5mM lead acetate	Yellow leaves, abnormal growth at shoot apex. Appearance of abnormal bud.
	5mM lead acetate and SSD1	Yellow leaves, abnormal growth at shoot apex. No bud seen.
Day 30	Control	Normal plant
	5mM lead acetate	Yellow leaves, abnormal growth at shoot apex. Curled leaves with abnormal bud.
	5mM lead acetate and SSD1	Yellow leaves, abnormal growth at shoot apex. Curled leaves with abnormal bud.

Solanum nigrum:

Time	Treatment	Phenotypic changes
Day 1	Control	Green healthy plants with green fruit, spotless leaves and stem
	5mM Lead acetate	
	5mM lead acetate and SSD1	
Day 7	Control	No visible changes
	5mM lead acetate	
	5mM lead acetate and SSD1	
Day 15	Control	Normal plant with no changes
	5mM lead acetate	Slight yellow leaves and curling of new young leaves.
	5mM lead acetate and SSD1	Slight yellow leaves
Day 20	Control	Normal plant
	5mM lead acetate	Yellow leaves with curling
	5mM lead acetate and SSD1	Yellow leaves with curling
Day 25	Control	Normal plant
	5mM lead acetate	Yellowing and prominent curling of young leaves
	5mM lead acetate and SSD1	Leaves getting dry
Day 30	Control	Normal plant
	5mM lead acetate	Leaves yellow, dry and curly.
	5mM lead acetate and SSD1	Leaves yellow, dry and curly.

Scanning Electron Microscopic Analysis of Plant Parts:

Control Plants:

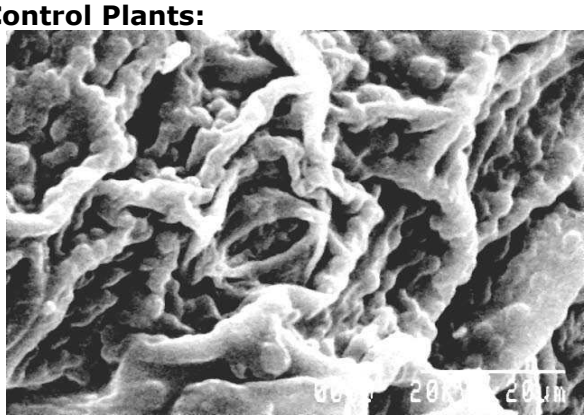


Fig.22: *Solanum nigrum* leaf (1000x)

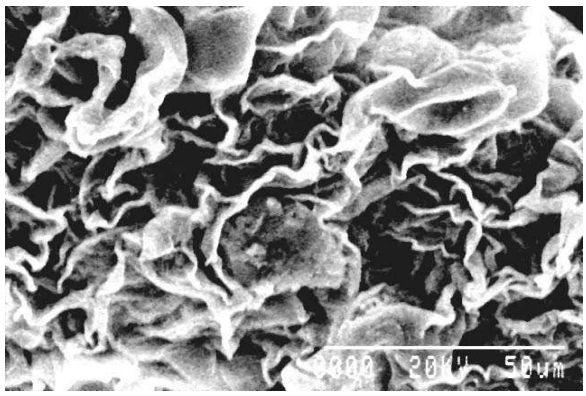


Fig.24: *Solanum nigrum* leaf (1000x)

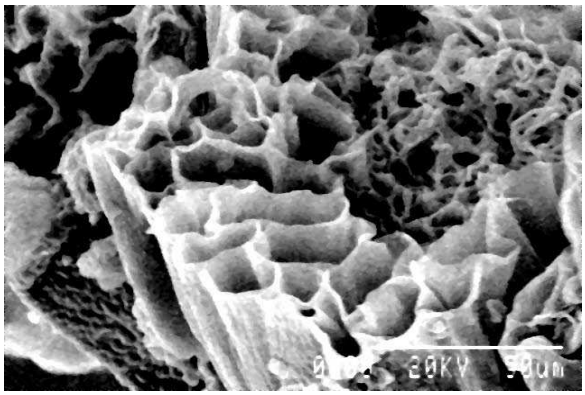


Fig.23: *Solanum nigrum* leaf (800x)

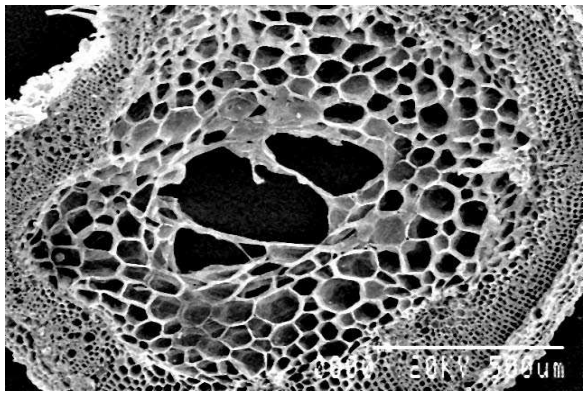


Fig.25: *Solanum nigrum* stem (800x)

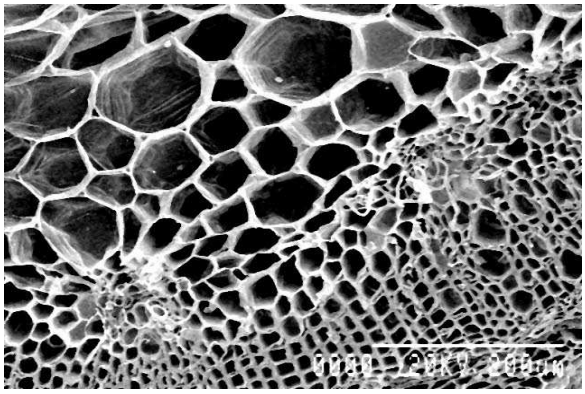


Fig.26: *Solanum nigrum* stem (1500x)

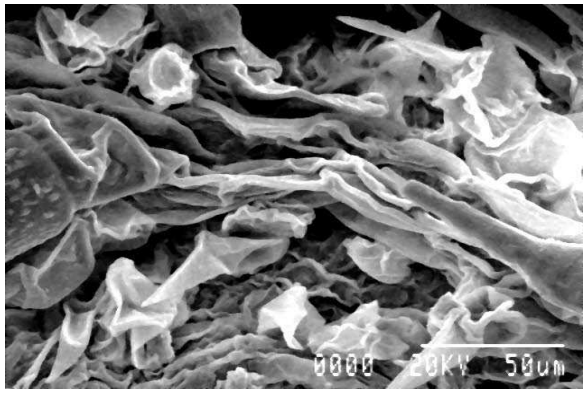


Fig.30: *Helianthus annus* leaf (500x)

Treated plants:

***Helianthus annus*:**

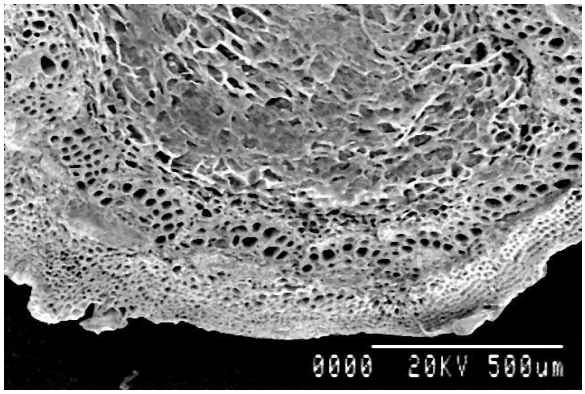


Fig.27: *Helianthus annus* stem (800x)

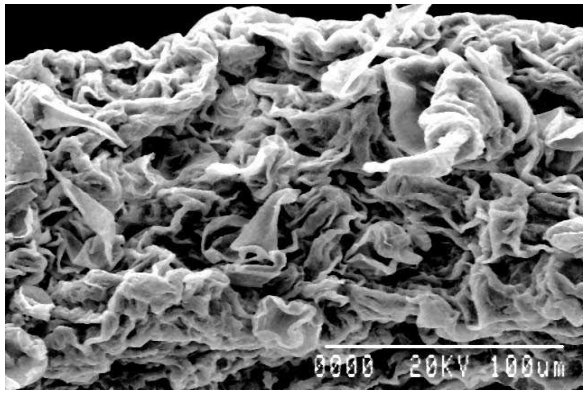


Fig.31: Leaf treated with lead (500x)

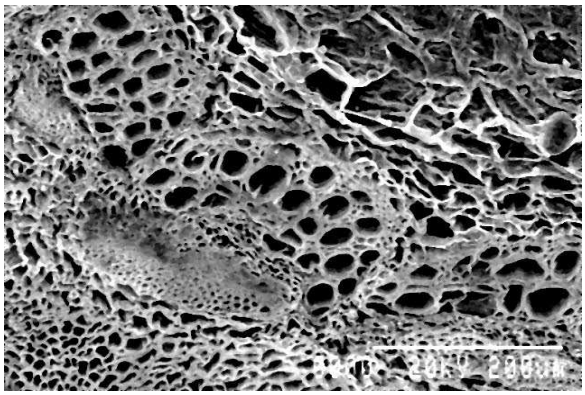


Fig.28: *Helianthus annus* stem (1500x)

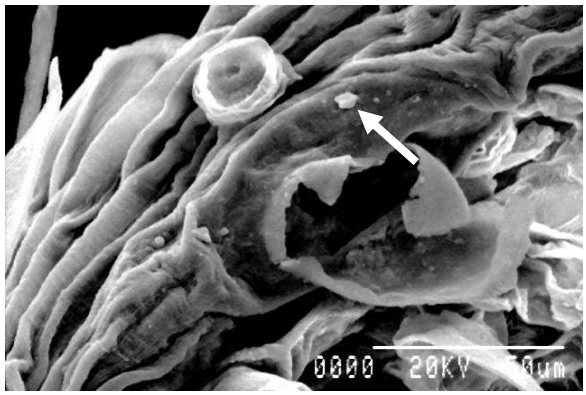


Fig.32: Leaf treated with lead (800x)

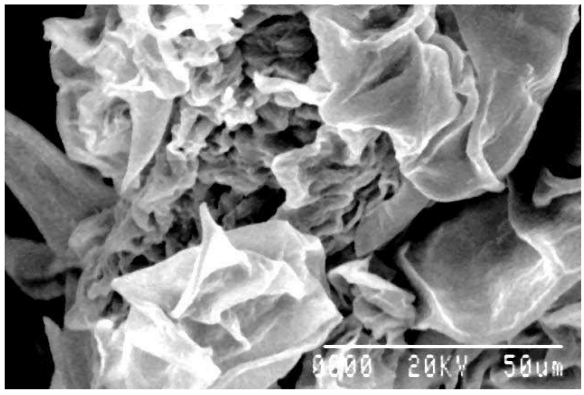


Fig.29: *Helianthus annus* leaf (1000x)

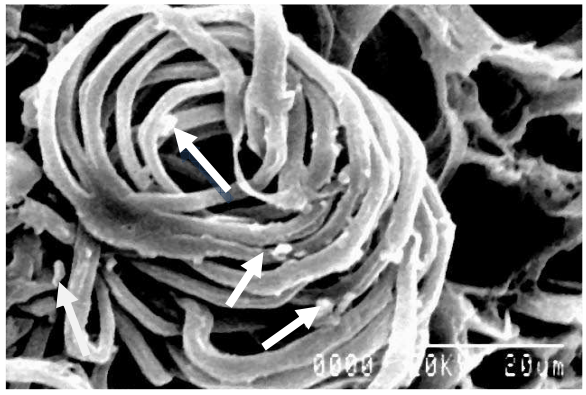


Fig.33: Stem treated with lead (1500x)

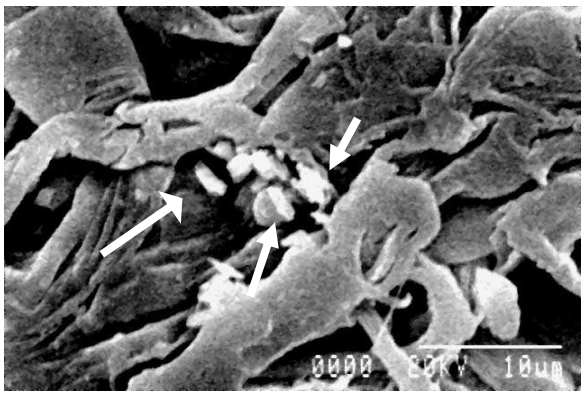


Fig.34: Stem treated with lead (3000x)

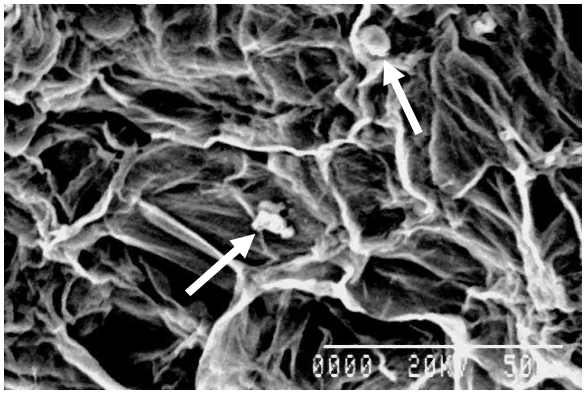


Fig.35: Stem treated with lead and bacteria (1000x)

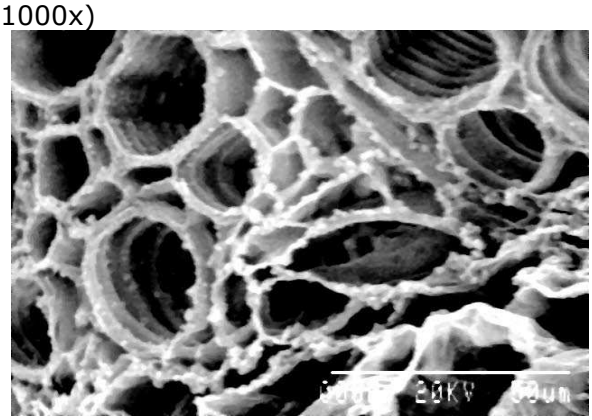


Fig.36: Stem treated with lead and bacteria (1000x)

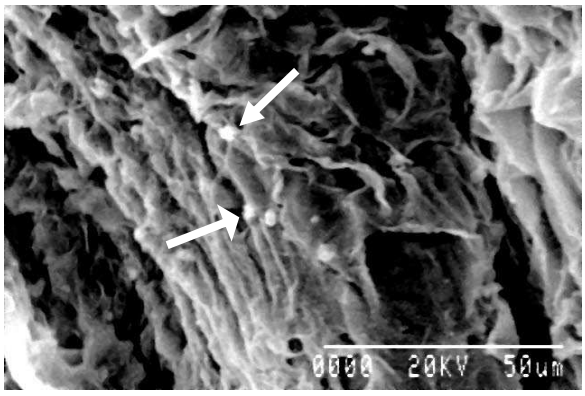


Fig.37: Leaf treated with lead and bacteria (1000x)

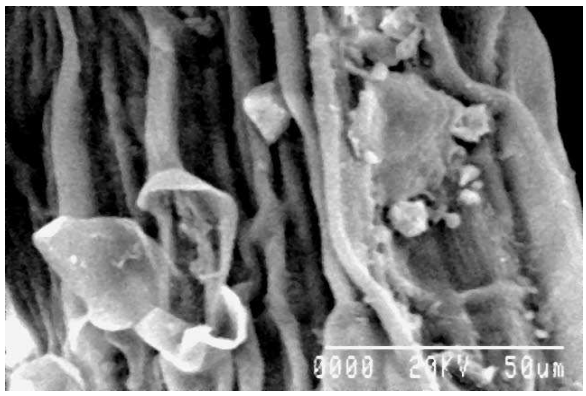


Fig.38: Leaf treated with lead and bacteria (1000x)

***Solanum nigrum*:**

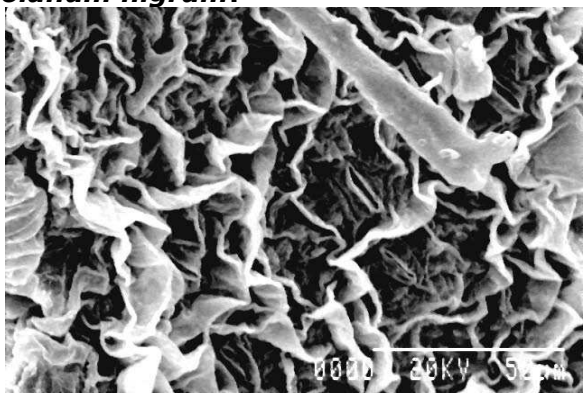


Fig.39: Leaf treated with lead (500x)

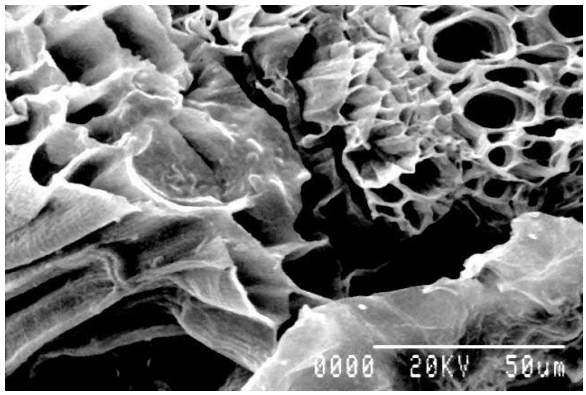


Fig.40: Leaf treated with lead (800x)

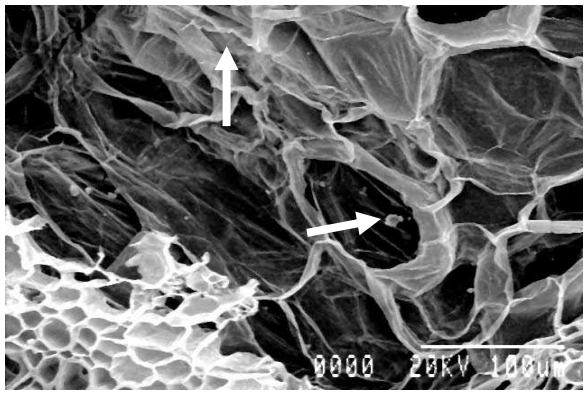


Fig.41: Stem treated with lead (500x)

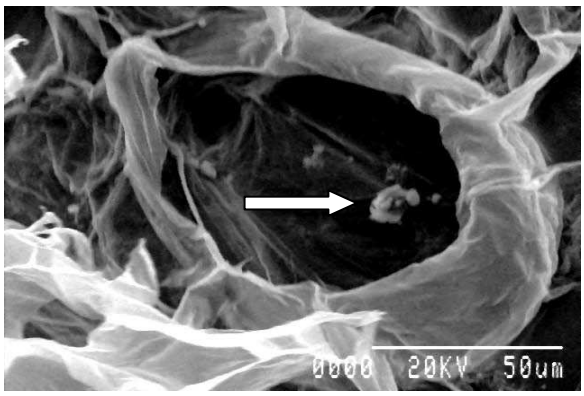


Fig.42: Stem treated with lead (800x)

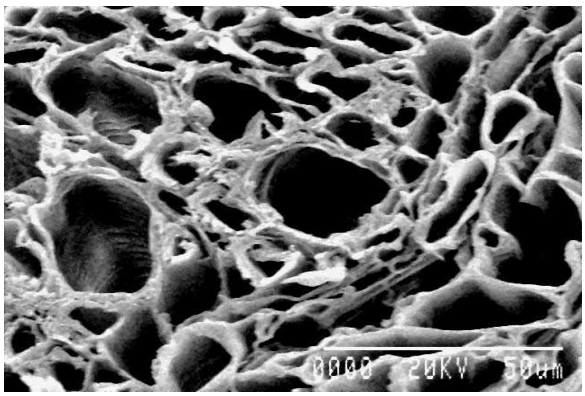


Fig.43: Stem treated with lead and bacteria (1000x)

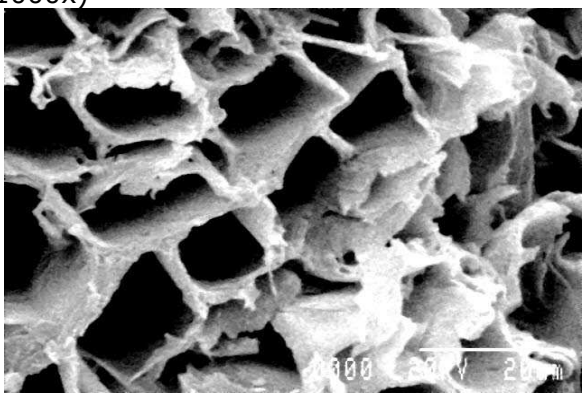


Fig.44: Stem treated with lead and bacteria (1500x)

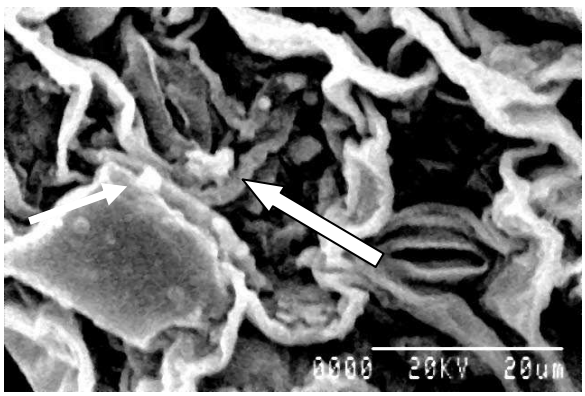


Fig.45: Leaf treated with lead and bacteria (2000x)

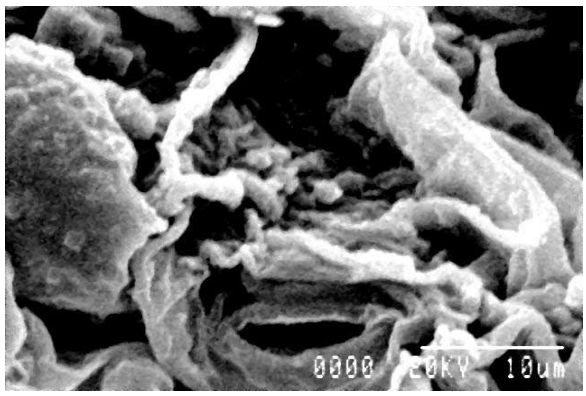


Fig.46: Leaf treated with lead and bacteria (3000x)

In this study, serial dilution of the soil sample was carried out and then pour plated over lead acetate supplemented LB agar. Dominant colonies, in terms of size were chosen to isolate heavy metal resistant strains of bacteria. Five such strains were obtained after growing the selected dominant colonies in 1mM lead acetate supplemented LB broth. Out of the five lead resistant isolates, a white colony, named SSD1, which selected from 10^{-5} dilution plate (Fig 5) was chosen for our study as it showed the highest growth. Biochemical tests performed with all the five isolates showed varying results (Table 3-6 and Fig 6-21). SSD1 was observed to be Gram negative endospore former. Biomass of SSD1 was found to decrease with increasing concentration of lead acetate (Table 2). Bacterial growth curve experiment performed with SSD1 showed nearly same growth curve pattern in presence or in absence of 5mM lead acetate but an extended lag phase of the treated culture (with Pb) was observed (Graph 1). Optimal conditions for the growth of SSD1 was found to be 30-37^{o}C and p^{H} 6-7 (Graph 2 and 3). Genomic DNA extraction of SSD1 was done. PCR amplification of 16s rRNA, sequencing and protein profiling of SSD1 will be our future aim of study.

The study also showed that a beneficial association, in terms of plant growth exists between SSD1, and the heavy metal resistant plants, *Helianthus annus* and *Solanum nigrum*. Results showed prominent high growth of leaf and stem in plants inoculated with SSD1, which indicates positive role of the bacteria in plant growth promotion even in a stressed environment (Graph 4-7). Distinct phenotypic changes were observed in both the plants 10 days after application of 10ml of (5mM concentration) lead acetate

solution. This showed the adverse effects of heavy metal intoxication as the bio absorption of lead occurs in the plants (Plate1 and Table 7).

Helianthus annus treated with 5mM lead acetate solution but without SSD1 inoculation showed more lead deposition in stem than in leaf. Plants treated with both lead acetate and SSD1 showed similar results (Fig 31-34). Inoculation with SSD1 showed less deposition of lead, which indicates that some of the heavy metals might had been accumulated by SSD1 (Fig 35-38). In case of *Solanum nigrum*, without any SSD1 inoculation and deposition of lead occured mostly in stem with negligible amounts in leaves (Fig 39-42). *Solanum nigrum* inoculated with SSD1 showed no deposition of lead in stem and a single instance of deposition in leaf. This indicates that SSD1 had negative effects on bioaccumulation capabilities of *Solanum nigrum* (Fig 43-46). This contrasts the result obtained for *Helianthus annus* as both SSD1 and the plant was observed to be working synergistically for remediation of lead. The mechanism of heavy metal uptake to different parts of the plants and the detailed synergistic mode of action of SSD1 and *Helianthus annus* needs further investigation. The plant-microbe beneficial interaction and mass cultivation at contaminated sites will be our future area of research.

Conclusion

The plants used for this study were able to grow in lead contaminated environment. Synergistic interaction was observed between the heavy metal tolerant bacterial isolate, SSD1 and *Helianthus annus*. But the interaction between *Solanum nigrum* and SSD1 showed negative effects as the bioaccumulation ability of the plant decreased significantly. Thus from this present study it can be concluded that a plant-microbe system can be very ideal for heavy metal remediation in contaminated sites, especially with *Helianthus annus* and the isolated bacteria.

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